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Oxidative DNA damage has been closely linked to cancer development. An active DNA repair system is critical to prevent the occurrence of mutations leading to carcinogenesis. It was the objective of this investigation to test the hypothesis that natural products such as flavonoids are able to stimulate the repair of oxidative DNA damage. For this purpose LNCaP prostate tumor cells were exposed to FeSO4 to induce oxidative DNA damage (8-hydroxydeoxyguanosine determined by HPLC). DNA repair was evaluated by following the decrease of oxidative DNA damage over time. We were able to demonstrate that in LNCaP cells exposed to naringenin (80 μmol/L) oxidative DNA repair activity was increased by 24% compared to media treated controls. RT PCR results demonstrated that the increase in DNA repair was associated with an increased mRNA expression of three BER repair enzymes important in the repair of oxidative DNA damage: 8-oxoguanine-DNA glycosylase 1 (hOGG1), apurinic/apyrimidinic endonuclease (APE) and DNA polymerase β (DNA pol-β). We observed the maximum stimulatory effect on mRNA expression at 24 hours of naringenin treatment. Currently additional flavonoids and in the near future flavonoid metabolites and breakdown products will be tested for their DNA repair-stimulatory activity.

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Introduction

Prostate cancer is a disease of aging. The oxidative stress hypothesis of aging postulates that oxidative damage to critical molecules accumulates over a life span leading to chronic disease and cancer. Reactive oxygen species are formed continuously in living cells as byproducts of normal cellular metabolism, as well as by exogenous sources. In case of a failure to remove DNA damage, mutations occur at a high rate and contribute to malignant transformation. A particular abundant lesion, 8-hydroxydeoxyguanine (8-OHdG), is highly mutagenic, yielding GC to TA transversions [1]. The base excision repair (BER) pathway is responsible for the repair of oxidative DNA damage. Removal of the damaged base by 8-oxoguanine-DNA glycosylase 1 (hOGG1) comprises the first step followed by apurinic/apyrimidinic endonuclease (APE) activity [2]. DNA polymerase β (DNA pol-β) activity is responsible to fill the gap created by the excision of 8-OHdG. There is in vitro evidence that some flavonoids such as myricetin and baicalin will stimulate DNA repair [3;4]. Flavonoid concentrations used in these in vitro experiments are usually higher than physiologically achievable. It was the primary objective of this study to investigate the DNA repair stimulatory effect of different flavonoids such as naringenin, apigenin, ECG and their metabolic transformation products. It was the secondary objective to investigate if the intracellular concentration of these flavonoids is physiologically achievable.

Body and Key Research Accomplishments

Task 1:

a) The concentration of iron sulfate was optimized to produce reproducible oxidative DNA damage. 200 µmol/L $FeSO_4$ was chosen for future experiments. At this concentration oxidative DNA damage was increased 4.6 fold compared to cells not treated with $FeSO_4$. At this concentration DNA damage is high enough to be measured reproducibly by HPLC without affecting the viability of the cells (Figure 2 and 3). At 200 µmol/L $FeSO_4$ we demonstrated that LNCaP cells were able to repair the oxidative DNA damage (Figure 4).

Figure 1. Oxidative DNA damage determined by HPLC in LNCaP P47 cells treated with different concentration of FeSO4 for 1 hour.

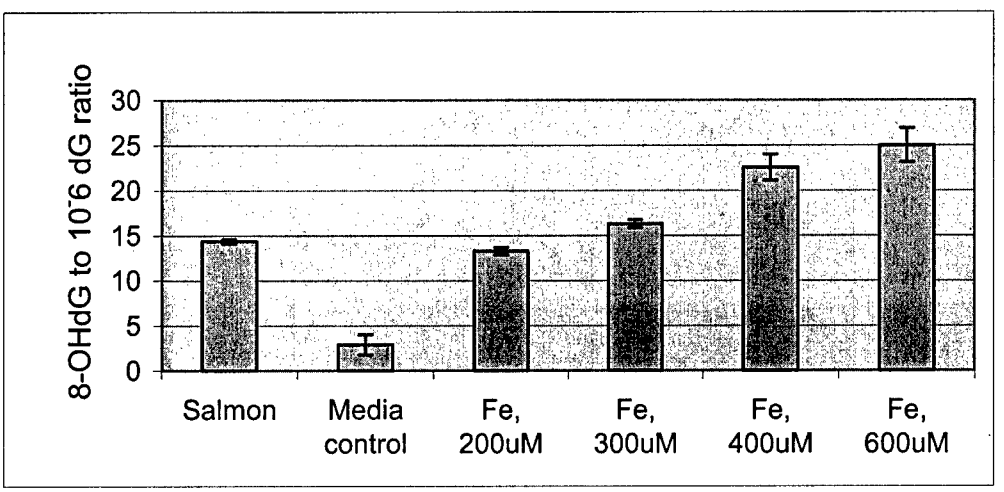


Figure 2. LNCaP P47 cell viability treated with different concentrations of FeSO4 and H_2O_2 for 1 hour and viability was tested immediately.

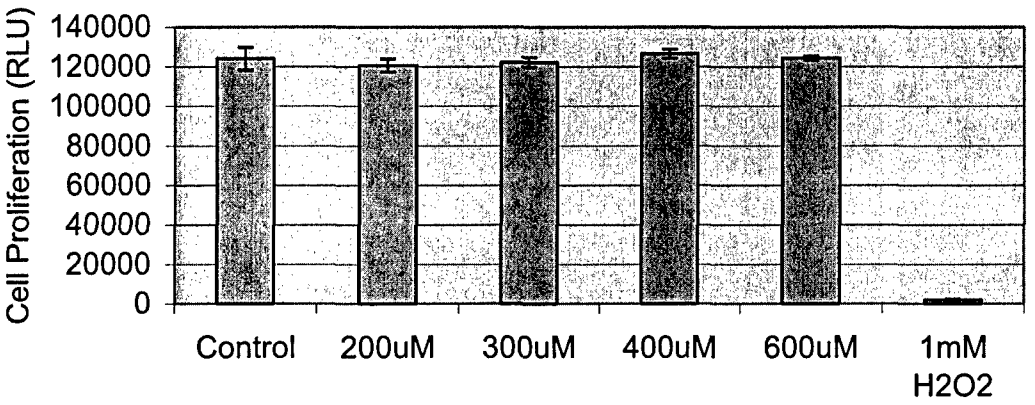


Figure 3. LNCaP P47 cell viability treated with different concentrations of FeSO4 and H_2O_2 for 1 hour and viability was tested after 24 hours.

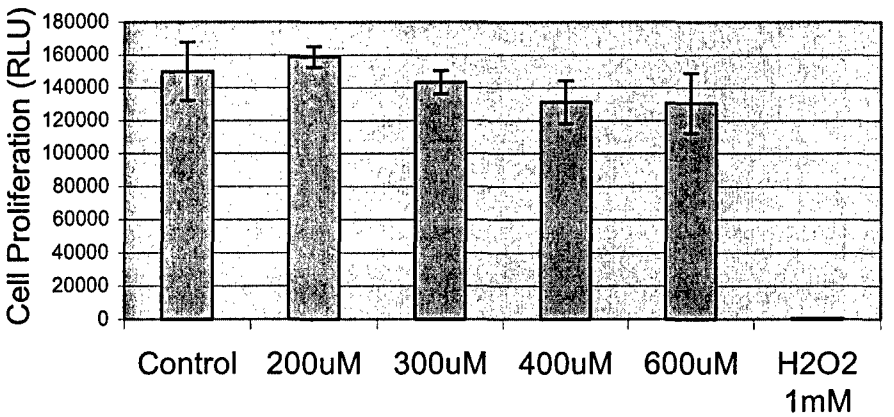
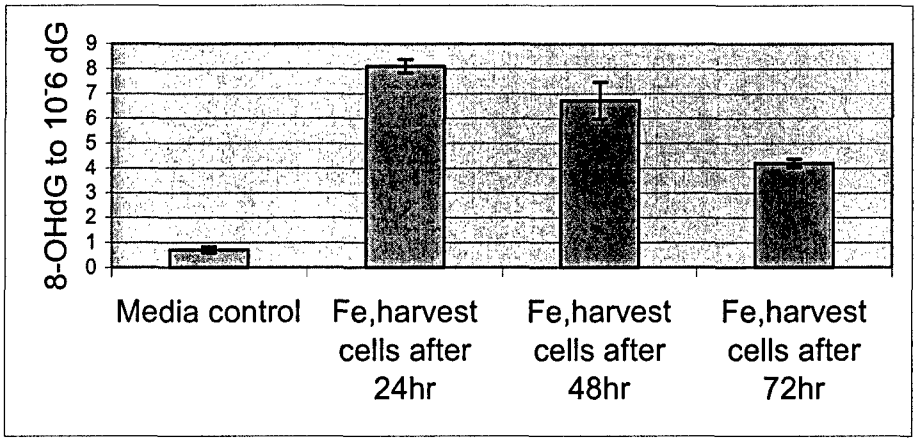


Figure 4. DNA repair in LNCaP P47 cells after FeSO4 (200 µmol/L) treatment for 24 to 72 hours.



Task 1:

b) To screen different flavonoids for their ability to stimulate DNA repair we screened the pH stability at pH 7 as in culture medium and antiproliferative effect. Based on these results we selected the following three flavonoids to continue the investigations on the stimulatory effect on DNA repair:
   -naringenin from citrus
   -apigenin from parsley
   -ECG from tea

These three flavonoids are representative of the flavanone and flavanol chemical subgroups. Unfortunately many compounds of the other flavonoid subgroups are not pH stable and will be degraded in cell culture experiments.

Figure 5. Stability of different flavonoids in cell culture medium at 0 to 29 hours.

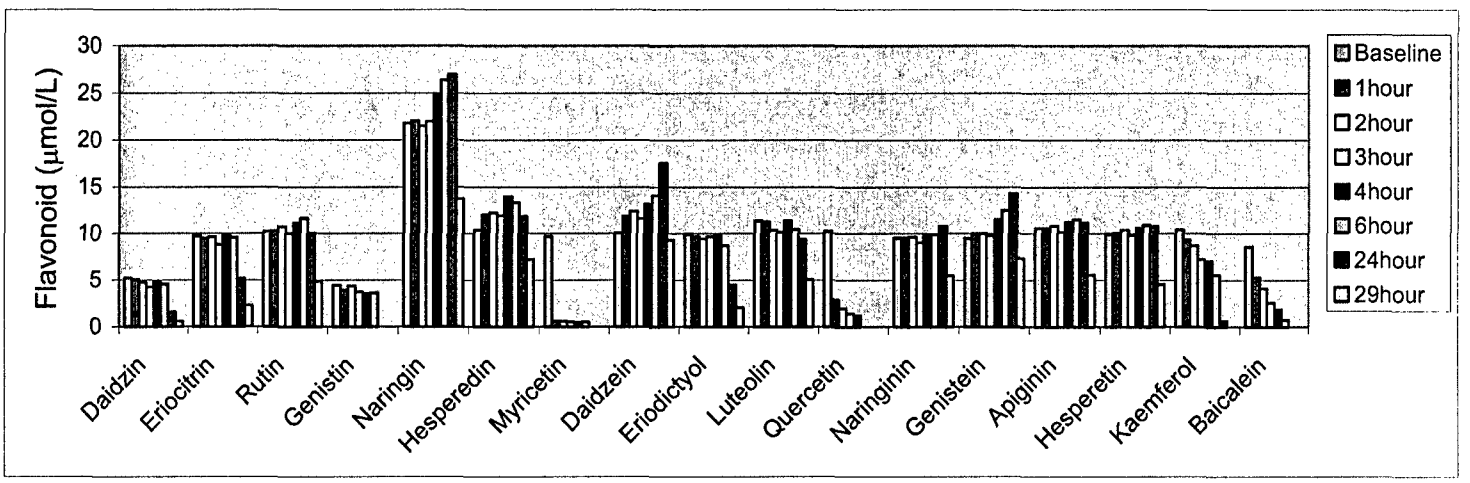
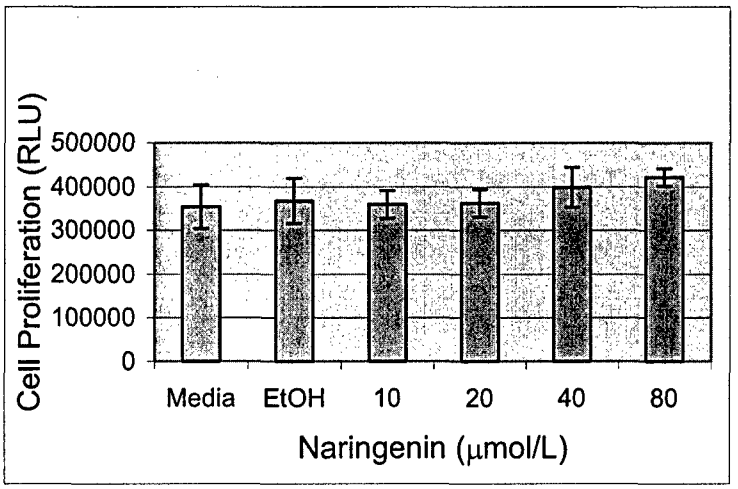


Figure 6. Antiproliferative activity of a) naringenin, b) ECG, c) apigenin and d) genistein at concentrations from 0 to 80 μmol/L.

a)



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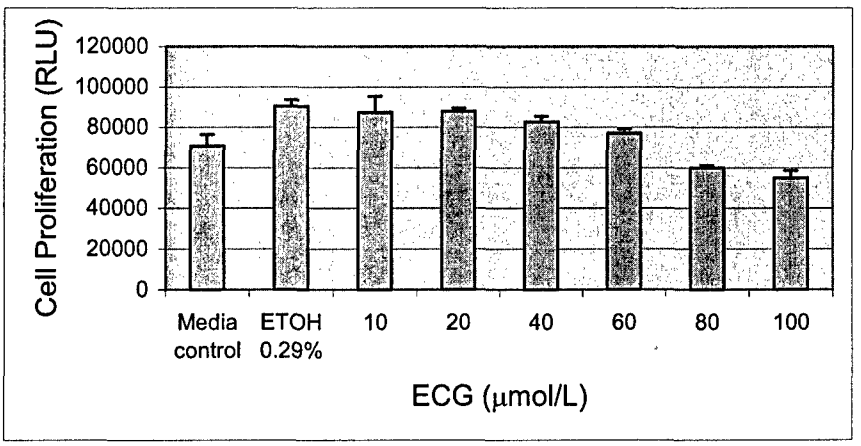

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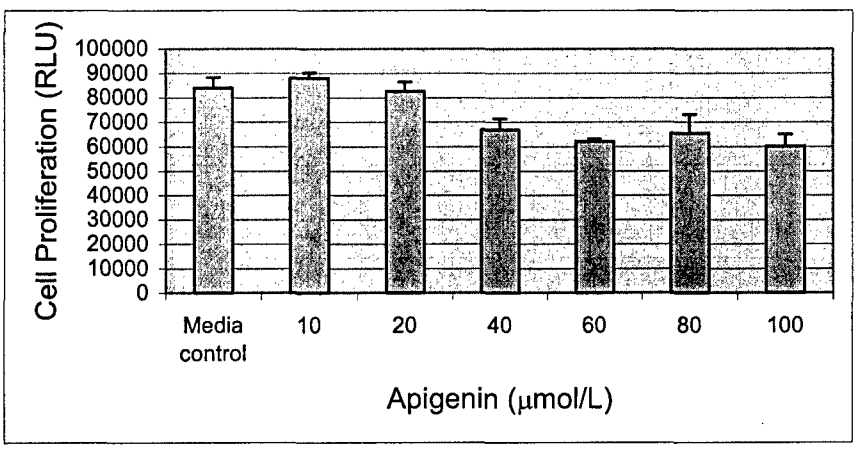

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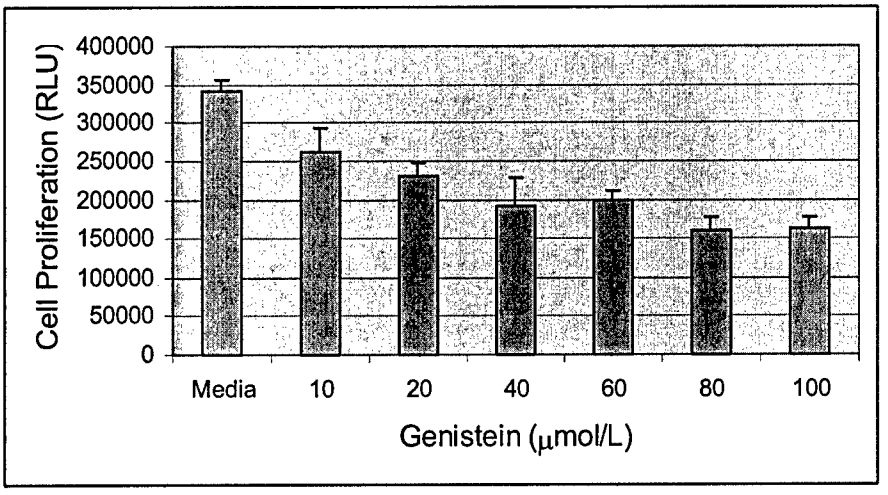
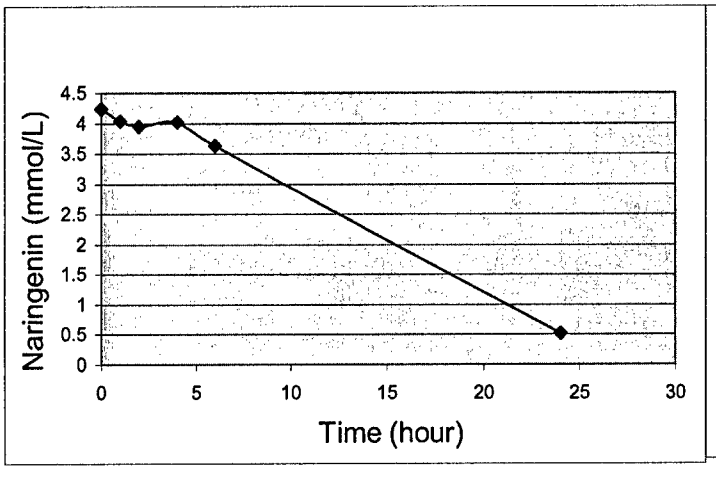


Task 1:

c) The intracellular concentration of naringenin was determined in LNCaP P47 cells cultured in RPMI 1640 medium after addition of 10 μmol/L of the individual flavonoid. The maximum intracellular concentration of naringenin was 40 pmol/10^6 cells. We currently are performing the same experiment using apigenin and ECG.

Figure 7. Decrease of naringenin in a) medium and uptake into b) LNCaP cells.

a)



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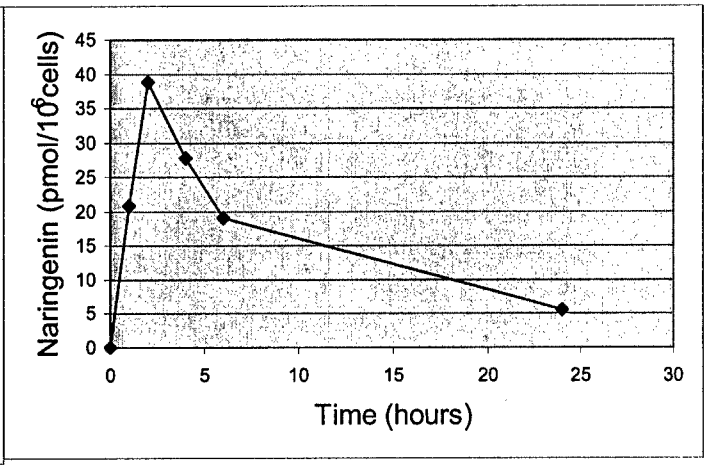


Task 1:

d) The DNA repair stimulatory effect of naringenin was determined in LNCaP cells exposed for one hour to FeSO4 (200 μmol/L). After removal of the iron, cells were treated with increasing concentrations of naringenin (10 to 80 μmol/L) for 24 hours. We demonstrated that naringenin stimulated DNA repair in turn leading to a decrease in oxidative DNA damage of 3-24% compared to media only treated cells (Figure 8). Using real time PCR we also demonstrated that mRNA expression of hOGG1, DNA pol-β and APE was increased at 8 hours compared to baseline and decreased at 24 hours. At 24 hours naringenin had the strongest stimulatory effect on mRNA expression of these BER enzymes (Figure 9). We currently are measuring the protein

concentration using Western blot to confirm that the increase in DNA repair is based on upregulation of these BER repair enzymes.

HOGG1, DNA pol-β and APE were determined using real-time PCR analysis. Total RNA was extracted using the RNAeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using Taq polymerase and oligo (dT) followed by PCR amplification using the following real-time PCR Assay-on-Demand custom-made kits (Applied Biosystems Inc., Foster City, CA): human OGG1: Hs00213454_m1; ; human polymerase beta: Hs00160263_m1 and human APE/ref-1: Hs00205565_m1. PCR reaction mix was prepared using Taqman Universal Master Mix (Applied Biosystems Inc., Foster City, CA), cDNA template in Rnase-free water, target assay mix or control assay mix. Samples were analyzed on the ABI 7700 (Taqman), which was available in the UCLA Sequencing and Genotyping Core Facility.

Figure 8. DNA repair stimulated by naringenin determined by the decrease in 8-OhdG/dG ratio in LNCaP cell DNA after treatment with 0 to 80 μmol/L naringenin.

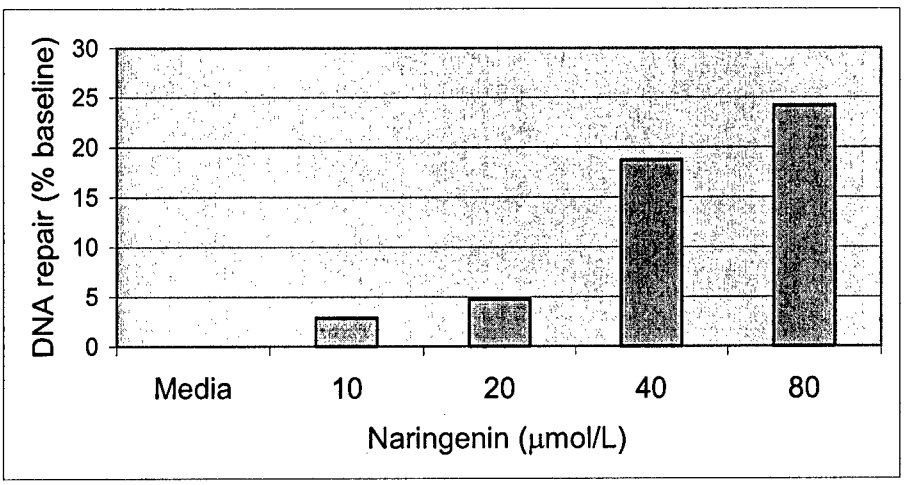
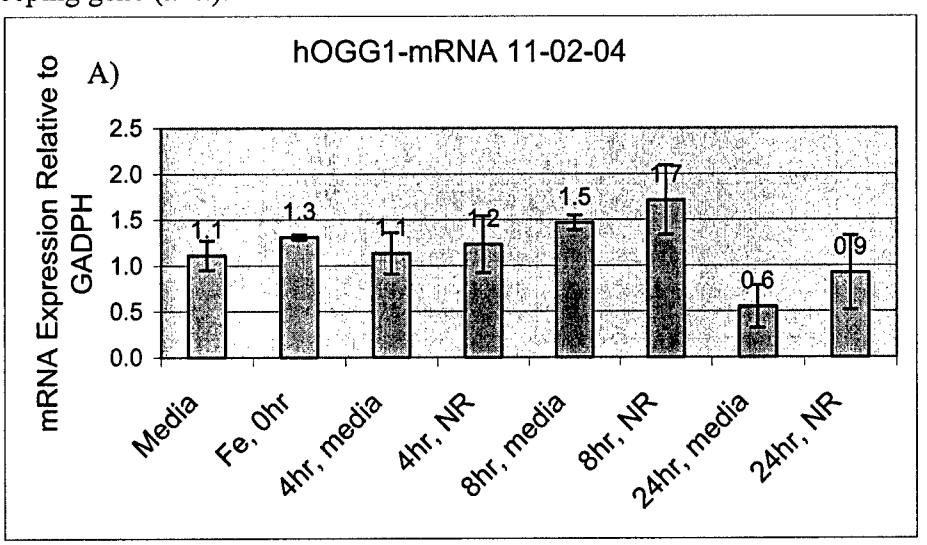
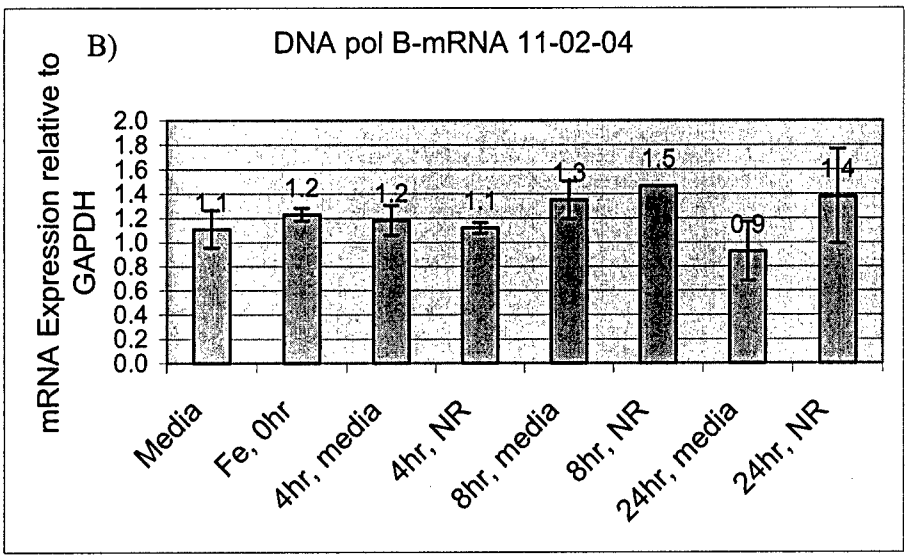
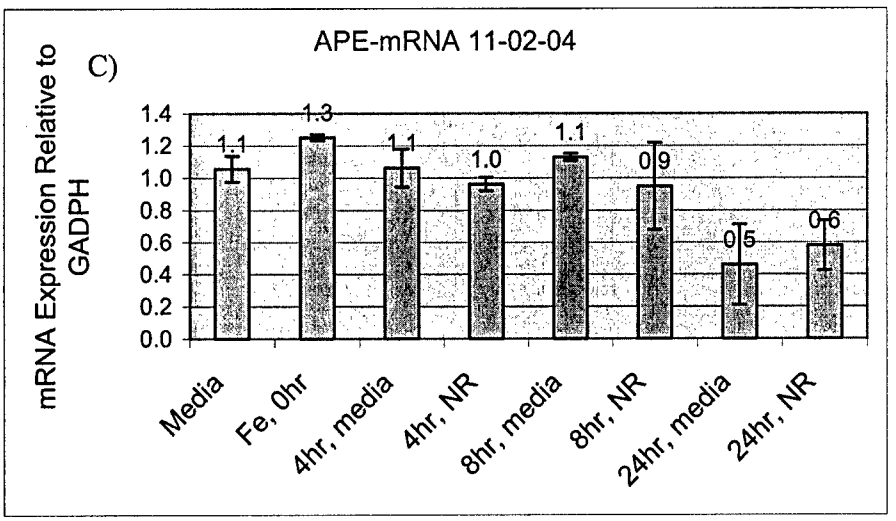


Figure 9. BER repair enzyme m-RNA expression determined by real time PCR after naringenin intervention for 0-24 hours. A) hOGG1; B) DNA pol-b; C) APE. HOGG1, DNA pol-β and APE mRNA expression in LNCaP cells was determined by RT-PCR and expressed in ratio to GAPDH housekeeping gene (n=2).







Task 2:

Possible synergistic effects of combinations of different flavonoids have not been investigated yet.

Task 3:

a) Commercially available human microsomes were purchased and exposed to naringenin. The incubation mixture was extracted with ethylacetate. Extracts were analyzed using HPLC after the incubation and peak area of naringenin and unknown new metabolite peaks were evaluated (Table 1). After incubation the peak area of naringenin was reduced by 53% and two new peaks were detected. Since human microsomes are very expensive and the amount of conjugated naringenin was not enough to perform any antiproliferative experiments we decided to use mouse liver homogenates to produce metabolites.

b) We have not been able yet to generate enough metabolites to test their DNA stimulatory activity.

Table 1: Incubation of human liver microsomes incubated with 10 μmol/L of naringenin.

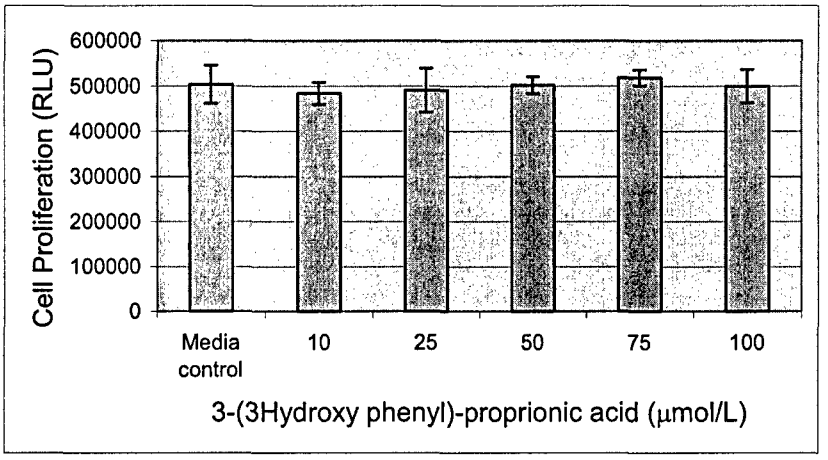
Retention Time	Compound	no microsomes	microsomes
		Peak area	
46.3	Unknown	4.2±1.3	61.9±0.6
48.7	Unknown	0.0	24.3±2.3
52.4	TRF Internal Std	31.4±0.7	33.8±1.3
57.4	Naringenin	317.9±4.5	151.8±0.8

Task 4:

The following phenolic acid breakdown products have been determined as intestinal metabolites from green and black tea, citrus and soy: 4OH-phenylacetic acid, 3-(3hydroxy-phenyl)-proprionic acid, homovanillic acid, 3,4dihydroxyphenyl acetic acid, hippuric acid, 2,4,6-trihydroxybenzoic acid; citrus: same phenolic acids, 3-hydroxyphenylacetic acid, 3-(4-hydroxy-3-methoxyphenyl)propionic acid, *p*-hydroxybenzoic acid. We tested the antiproliferative activity of 3-(3hydroxy-phenyl)-proprionic acid, 3,4dihydroxyphenyl acetic acid and 4-hydroxyphenyl acetic acid. We demonstrated a significant inhibition of cell proliferation at 24 and 48 hours at 75 μmol/L for 3.4dihydroxyphenyl acetic acid (Figure 10).

Figure 10. Antiproliferative activity of a) 3-(3hydroxy-phenyl)-proprionic acid, b) 3,4dihydroxy phenyl acetic acid and c) 4-hydroxyphenyl acetic acid at 10 to 100 μmol/L for 24 hours.

a)



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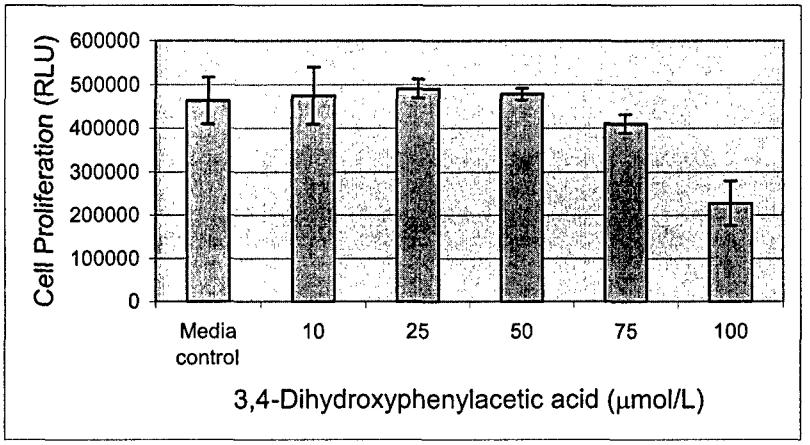

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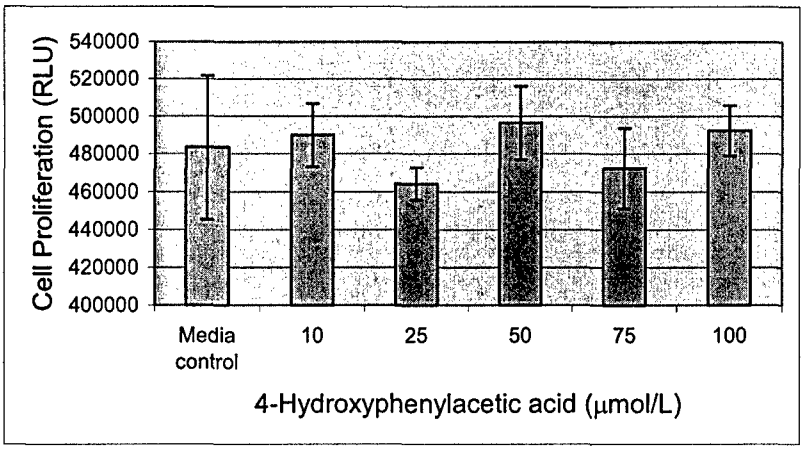

Reportable Outcomes:

One abstract was submitted to the 2005 Conference of the Federation of Experimental Biology: Susanne M. Henning, Kun Gao*, Anlong Xu*, and David Heber; 2005. The Citrus Flavonoid Naringenin Stimulates DNA Repair in Prostate Cancer Cells

Conclusions:

We were able to demonstrate that naringenin has DNA stimulatory activity in LNCaP cells. This increase in DNA repair was based on an increase in three DNA repair enzymes such as hOGG1, DNA pol-b and APE. Currently other flavonoids and in the near future flavonoid metabolites and breakdown products will be tested for their DNA repair-stimulatory activity.

Future Studies:

Task 1a) test 5a-dihydrotestosterone, use normal prostate cells
Task 1c) establish intracellular concentration for apigenin and ECG.
Task 1d) other flavonoids such as apigenin and ECG are being tested for their DNA repair-stimulatory activities.

Task 2) screen combinations of flavonoids for their synergistic activity

Task 3) produce large scale amounts of conjugates. Determine identity of conjugates using LC/MS. Screen conjugates for DNA repair-stimulatory activity

Task 4) screen breakdown products for DNA repair-stimulatory activity

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3. Morel,I., Abalea,V., Cillard,P., and Cillard,J. (2001) Repair of oxidized DNA by the flavonoid myricetin. *Methods Enzymol.*, **335**, 308-316.

4. Chen,X., Nishida,H., and Konishi,T. (2003) Baicalin promoted the repair of DNA single strand breakage caused by H2O2 in cultured NIH3T3 fibroblasts. *Biol.Pharm.Bull.*, **26**, 282-284.

Appendices:

Abstract submitted to the 2005 Conference of the Federation of Experimental Biology:

The Citrus Flavonoid Naringenin Stimulates DNA Repair in Prostate Cancer Cells

Susanne M. Henning, Kun Gao*, Anlong Xu*, and David Heber. Center for Human Nutrition, University of California, Los Angeles, CA 90095; *Key Laboratory of Genetic Engineer of MOE, Department of Biochemistry, College of Life Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou, Guangdong, 510275 China

As part of a systematic study of the effects of phytochemicals beyond anti-oxidation on cancer, we investigated whether naringenin (NR), a citrus flavonoid, will stimulate DNA-repair following oxidative damage in LNCaP human prostate cancer cells. Cells were cultured in serum free RPMI 1640 media and were treated with 200μM ferrous sulfate ($FeSO_4$) for 60min to induce oxidation. After changing the media to remove the ferrous sulfate stimulated oxidation, cells were treated with NR (10-80 μM) for an additional 24hrs. DNA was extracted, digested and the ratio of 8-OhdG to dG was determined using an Agilent 1100 HPLC system with ESA Couochem II detector. NR concentrations were measured in the media and cell extracts by HPLC. When exposed to 10-80μM NR in the media, the ratio of 8-OhdG/dG decreased by 3-24% compared to control cells without NR. NR content in the medium varied from 27-190 μM and there were to 4-28 μmol/mg protein contained in the prostate cancer cells. A linear relationship was found between NR concentration and the decrease of 8-OhdG/dG ratio demonstrating that a flavonoid found in citrus fruits (NR) stimulates DNA repair in prostate cancer cells at physiologically achievable concentrations. Therefore, citrus fruits may exert part of their cancer preventive effect demonstrated in population studies through stimulation of DNA repair, which prevents mutagenic changes leading to carcinogenesis.